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CHARACTERIZATION OF COMPONENTS OF THE TOLL/NF-kB SIGNAL TRANSDUCTION PATHWAY: IMPLICATIONS FOR HOST DEFENSE

Jesse A. Flaxenburg

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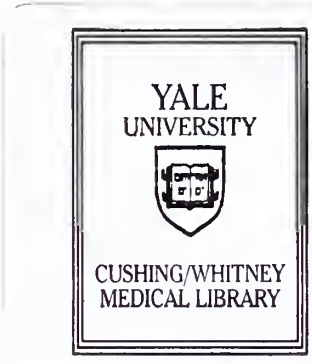
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Characterization of Components of the Toll/NF-κB Signal Transduction Pathway: Implications for Host Defense

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
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By
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CHARACTERIZATION OF COMPONENTS OF THE TOLL/NF-κB SIGNAL TRANSDUCTION PATHWAY: IMPLICATIONS FOR HOST DEFENSE

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Abstract: The transcription factor NF-kB plays a central role in the initiation of innate and adaptive immune responses via regulation of genes that encode various immunomodulatory proteins including cytokines and costimulatory molecules. NF-kB has been shown to become induced via a signal transduction pathway initiated by the activation of the Toll receptor. Here we seek to describe the function of a putative member of that pathway, the interleukin-1 receptor-associated kinase (IRAK). IRAK is a known member of a unique family of kinases referred to as serine/threonine innate immunity kinases (SIIK). Additionally, we report evidence explaining the function of the Toll receptor. In order to assess the function of IRAK we constructed a chimeric molecule consisting of the extracellular portion of the CD4 receptor and IRAK, which potentiates the ability of IRAK to induce NF-kB. Additionally, a kinase deficient form of this activated IRAK molecule was created to assess the role of phosphorylation in the function of IRAK. In order to test the role of the NH2-terminal death domain region in protein-protein interactions involved the NF-κB pathway we created death domain-deleted chimeric molecules as well. We then transfected human embryonic kidney epithelial cells containing a luciferase reporter gene driven by an NF-κB responsive promoter. Here we report that IRAK activates NF-κB and that activation does not absolutely require kinase activity. NF-κB activation by death domain-deleted molecules was potentiated by dimerization indicating a role for the death domain region in protein-protein interaction. CD4/IRAK-induced NF-κB activity was inhibited by a dominant negative version of known signal transducer TRAF6 indicating a downstream position of this protein. Additionally we report that Toll activation of NF-kB is potentiated by dimerization. Thus, we conclude that IRAK is a SIIK involved in the Toll/NF-kB pathway upstream of the TRAF6 protein. The function of IRAK in this pathway is not solely mediated by kinase activity. Additionally, we conclude that the Toll receptor may function in a dimer suggesting the putative ligand is likely generated by an endogenous process.

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1. Introduction

The mechanism by which an organism defends itself against infectious agents has occupied the thoughts of countless researchers over the last 200 years. Over that time it has become apparent that the human immune response is two-pronged. One facet is a specific response, known as an adaptive immune response, designed to protect against a particular pathogen. The other is innate immunity. By contrast, innate immunity has the potential to protect against a wide range of pathogens. Much research in the field of immunology today is directed toward elucidating the interplay between these two responses.

1.1 Adaptive Immunity

Adaptive immunity is dependent upon the recognition of specific pathogens. This is accomplished by the production of antibodies by activated B-lymphocytes. Antibodies are encoded by specialized regions of DNA which are capable of somatically recombining.[1] This act of recombination allows B cells to elaborate antibodies adept at recognizing many pathogen-associated antigens with a relatively few number of genes.

Nevertheless, the induction of an adaptive immune response requires the activation of other elements of the immune system. Upon initial infection with a microorganism, host cells specialized in the initiation of an immune response become activated. These cells are referred to as professional antigen presenting cells (APC) and consist of essentially three types of cells with somewhat differing functions: dendritic cells, macrophages and, in some cases, B-cells.

The initiation of specific immunity begins with the presentation of foreign antigen on the surface of APCs. This is accomplished with the help of glycoproteins encoded for by genes of the major histocompatabilty complex. Thus, the proteins are termed MHC proteins. Upon encountering a microorganism, APCs ingest the pathogen into lysosomal and endosomal compartments where foreign peptides are digested. The vesicles which contain these fragments then fuse with vesicles containing MHC class II proteins. The peptides then bind the MHC class II molecules in a specific manner at which point they are transported to the surface of the APC.[2]

It is at this point that naïve T lymphocytes recognize the MHC/peptide molecule. This interaction occurs not at the primary site of infection but at peripheral lymphoid organs such as Peyer's patches in the digestive tract and the tonsils.[3] T cells possess receptors that are generated in much the same way antibodies are. Thus, each organism has the ability to produce a vast repertoire of T cell receptors (TCR) capable of recognizing an enormous variety of MHC/peptide complexes. Additionally, there exist a number of other families of molecules which mediate T cell/APC interaction. These include selectins, vascular addressins, integrins (specifically leukocyte function-associated antigen-1) and intercellular adhesion molecules (ICAMs) which contribute to homing and migration of lymphocytes as well as APC binding, thus ensuring that T cells reach their proper target cell.[4,5]

The process of priming, activating naïve T cells into effector T cells, requires more than recognition by a somatically recombined TCR of its MHC/peptide ligand. In addition to the presentation of the CD4 molecule, which is required for T cell recognition of MHC class II/peptide molecules on the surface of APCs, T cells must also recognize a

second signal from APCs. This signal, which has been termed costimulation, is mediated through the expression of glycoproteins in the B7 family. The receptor on the surface of T cells responsible for the recognition of this costimulatory ligand is CD28. Several experiments have shown that CD28 cross-linking is an essential feature of T cell activation and that the absence of such recognition, even in the presence of a specific MHC/TCR interaction will lead to a quiescent T cell state known as anergy.[6,7,8,9] Anergic T cells are refractory to activation.

Once the naïve T cell binds both the MHC class II/peptide and the costimulatory molecule on the surface of the APC, it becomes activated. At this point the T cell begins to elaborate a growth factor known as interleukin-2 (IL-2) which triggers clonal proliferation of T cells bearing the specific TCR of the activated clone. It is at this point when the CD4 T cell will further differentiate into a TH1 or TH2 cell. TH1 cells direct an inflammatory response by secreting macrophage-activating cytokines interferon-γ (IFN-γ), granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor-β (TNF-β). TH2 cells trigger a humoral immune response by elaborating B-cell activating cytokines IL-4, IL-5 and IL-6. Upon signaling with these cytokines, B-cells are activated to differentiate into specialized antibody-secreting cells. Additionally, the predominance of a specific cytokine in the TH2 pathway is responsible for signaling a specific type of antibody response, e.g. IgG, IgE, etc.

Note that this has been a rather cursory look at how the human organism develops specific effector T cells that can rapidly initiate an adaptive immune response upon further inoculation with a specific offending organism. The key event in this pathway, for our purposes, is the expression of the costimulatory molecule by the APC. While the

mechanism for the prevention of autoimmunity has been historically cited as the process of negative selection of autoreactive T cells in the thymus, there is evidence to support the notion that many T cells capable of recognizing self-peptides presented by MHC molecules reach the circulation. Additionally, under this hypothesis, there exists no mechanism to delete T cells bearing receptors capable of recognizing innocuous non-self peptides presented in the context of MHC. Thus, there must exist another mechanism for the prevention of autoimmunity and destructive hypersensitivity reactions, both of which are far less prevalent in the human population than that which would be predicted without such a mechanism in place. As we shall see, the presentation of costimulatory molecules provides that mechanism. Additionally, the method by which organisms induce costimulation provides a glimpse into the much sought-after link between adaptive and innate immunity.

1.2 Innate Immunity

In contrast to the highly specific nature of adaptive immunity, there exists a mechanism of host defense that acts in a more immediate and general fashion. This has been termed innate immunity. Host organisms encounter countless challenges from invading microorganisms in a constant manner. Yet, a disease state arising from infection occurs only occasionally. Most foreign invaders are destroyed within a short period of time by this system of innate immunity.

One important method of host defense is the activation of the complement pathway. Although there exist three pathways of complement activation, the endpoints are similar. The result of complement activation is three-pronged. The production of

peptides C3a, C4a, and C5a result in an inflammatory response and act as chemotactic agents for host phagocytic cells.[10] Additionally, complement is responsible for the opsonization of pathogens. Because macrophages possess complement receptors, engulfment and destruction of opsonized microorganisms can occur. Lastly, the end result of the complement pathway is the production of a membrane attack complex consisting of the components C5b, C6, C7, C8 and C9.[11] This complex has the ability to create a pore in the membrane of attacking pathogens thus directly leading to their demise.

While the endpoints of complement activation are quite similar there are, as I have stated before, three mechanisms for activating this pathway. One is the classical pathway. This pathway is activated via antibody-antigen interaction and thus can be classified as part of an adaptive immune response. The second method is the lectin pathway which is initiated by the cross-linking of the mannose binding protein to certain carbohydrate structures on microorganisms. The last pathway is the alternative pathway. The alternative pathway is initiated with direct interaction between an activated complement component and the surface of a pathogen.[12] Both the lectin and alternative pathway share features consistent with innate immunity.

In the alternative pathway of complement activation, spontaneously cleaved C3b binds to the surface of a microorganism where it attaches to factor B. Factor B is then cleaved to Bb by factor D. Through a series of events, the C3b/Bb complex acts as a C3 convertase to initiate the complement cascade, which results in opsonization, the production of the potent inflammatory peptide C5a and the activation of terminal complement components. The possibility of alternative complement activation against

self is tightly regulated by the presence of complement control proteins CR1, H, MCP and DAF on the surface of host cells. These proteins disrupt the C3b/Bb complex preventing completion of the complement cascade.[13]

The lectin pathway, however, initiates a cascade, which is very similar to that of the classical pathway. The key step is the production of one of a family of proteins referred to as collectins. An example of a collectin is mannose-binding protein (MBP) which can recognize mannose residues which tend to be more accessible on bacterial surfaces compared with those of vertebrates. MBP normally exists at low levels in the serum but is quickly upregulated with infection. MBP is structurally similar to the C1q protein, which is responsible for the initiation of the classical complement pathway. Like C1q, MBP can act as an opsonin or initiate the complement pathway which eventually results in the production of proinflammatory peptides or the activation of the membrane attack complex. As we shall see, MBP is known as an acute phase response protein (APR) whose elaboration is elegantly tied to innate immune mechanisms.[14,15]

Another important mechanism of innate immunity is the function of host macrophages. Macrophages act as the primary agent of innate immunity in three important ways.[13] First, through the use of surface receptors they are able to recognize opsonized (Fc and complement receptors) pathogens or many microbial structures (mannose receptor, scavenger receptor, CD14). This recognition leads to engulfment and destruction of microbes through the production of oxygen radicals. Secondly, the interaction between a macrophage and pathogen leads to the secretion of cytokines. These intercellular signals provide valuable chemotactic instruction to cells of the immune system as well as initiating an inflammatory response. Lastly, macrophages are

important in antigen presentation and costimulation which leads ultimately to the induction of an adaptive immune response.

We have already seen how the presentation of costimulatory molecules is necessary for the initiation of an adaptive immune response. What is less obvious is the effect of cytokine secretion by activated macrophages on the initiation and potentiation of adaptive immunity. Of the myriad of mediators released by the macrophage in response to an invading organism perhaps the most important and best studied molecules are IL-1, IL-6, IL-8 and TNF-α.

IL-1 is primarily a potentiator of the inflammatory response. By activating vascular epithelium at the cellular level, IL-1 increases the ability of effector cells to reach the nidus of infection. Additionally, IL-1 can activate lymphocytes and, by acting on the hypothalamus, increase the set point of basal body temperature resulting in fever. Importantly, IL-1 potentiates the transcription of IL-6.[16]

IL-6 is an important activator of antibody production. Thus, there is a direct link between innate and adaptive immunity via this molecule. In addition, IL-6 is chiefly responsible for inducing hepatocytes to produce the acute phase reactants.[17] We briefly visited the APRs before with MBP. Additional APRs include serum amyloid protein, the clotting protein fibrinogen, and C-reactive protein which, like MBP can bind to pathogens in a fashion similar to antibody and either cause opsonization or initiation of the complement cascade.[18] The acute phase reactants thus provide a challenged organism with a window of opportunity during which time an adaptive immune response can be mounted.

IL-8 is sometimes referred to as a chemokine because its main function is to serve as a chemoattractant for various leukocytes. Thus, the mediators of the immune response are directed to the proper location.

TNF-α is a potent molecule capable of initiating an increase in vascular permeability which allows for the extravasation of antibody, complement and effector cells. In this way it can be seen as a potentiator of the adaptive immune response. Taken to its extreme, the systemic action of TNF-α can cause septic shock and disseminated intravascular coagulation (DIC). [16]

The result of this barrage of cytokines is the inflammatory response. The site of infection is quickly made accessible to various components of both adaptive and innate immunity ranging from complement and antibody to APRs to effector cells. Additionally, systemic ramifications play a part in infection control. The elevation of host core temperature retards microbial growth patterns. Providing another link between innate and adaptive immunity, this initial induction of cytokines is responsible for systemic leukocytosis. The rapid influx of neutrophils is important for both adaptive and innate immunity.[13]

1.3 Pattern Recognition

The mechanism by which triggers of innate immunity confer an adaptive immune response has been extensively studied in the past several years. Specifically, several questions have spurred an interest in the scientific community. How does the human immune system discriminate APCs presenting self and innocuous nonself antigen from those that present antigens belonging to infectious agents? How does the human immune

system compare to those of evolutionarily older organisms? Can we learn about the function of human immunity through the study of ancient systems of defense? Are there legitimate targets for improved manipulation of the human immune system in the fight against disease?

It has been proposed that the immune system of vertebrates can delineate the origin of antigens and direct an appropriate immune response via the action of a non-clonal system of receptors. When these receptors bind their respective ligands, a signal transduction cascade leads to the expression of costimulatory molecules and the production of effector cytokines.[19,20,21]

Although adaptive immunity occurs only in vertebrates, all multicellular organisms possess receptors whose function is host defense.[22] The ligands for these receptors are molecular patterns that are uniquely microbial in origin. Termed pathogen associated molecular patterns (PAMPs) they are necessarily invariant in their expression. Without the ability to alter the structure of such molecules, which would result in deleterious implications for their survival, pathogens lack the ability to evade detection by this novel class of receptors, termed pattern recognition receptors (PRR).[19,20,21] The activation of these receptors then becomes a means to alert the host organism that an infectious agent is present. Thus, there exists a mechanism for delineating self and innocuous nonself, which do not express PAMPs, from infectious nonself, which necessarily does.

Such a receptor has long been known to exist in the invertebrate *Drosophila*. Although the Toll receptor was originally discovered as a protein important in the establishment of a dorsal-ventral pattern in the *Drosophila* embryo[23,24] recent advances

have implicated it in host defense.[25,26] Specifically, the activation of the Toll receptor induces the production of the potent antifungal peptide drosomycin.[27] Recently, the human homologue of the Toll protein was cloned.[28] This protein is remarkably similar to its counterpart in *Drosophila* in both structure and function. Both are type I transmembrane proteins with an extracellular leucine-rich region (LRR), a cytoplasmic domain homologous to that of the human interleukin-1 receptor (the Toll/IL-1 receptor domain or TIR). Additionally, both were shown to signal through the NF-κB pathway. Induction of the human Toll receptor was shown to induce the expression of NF-κB controlled genes IL-1, IL-6 and IL-8. Perhaps most intriguing was that cross-linking Toll induced the expression of the costimulatory molecule B7.1.[28] Recent evidence now implicates the Toll receptor as the deficient protein in the LPS^d mouse, suggesting that lipopolysaccharide (LPS) is involved in the activation of Toll.[29]

With the discovery of the Toll protein and its implications in the invertebrate and vertebrate immune responses, an important link has been established. We now have two organisms, separated by hundreds of millions of years of evolution, that possess a virtually identical mechanism of innate immunity. Additionally, it has been shown, through the induction of B7.1 by cross-linking human Toll, that vertebrates have evolved an adaptive immune response which elegantly interacts with the evolutionarily older innate immunity. This adaptive immune response critically depends upon the first step, the binding of the PRR with its associated PAMP. Without this event, there is no signal of a foreign invader and the effector cells of adaptive immunity are not marshaled. Tolerance is induced. Thus, because of its central position in host defense, the Toll/ NF-

κB pathway has become the target of intense scrutiny. It is hoped that, through a greater understanding of this pathway, advances can be made in the treatment of human disease.

1.4 The NF-κB Pathway

NF-κB, a dimer consisting of members of the rel family of proteins, is a transcription factor that was first described in 1986 in reference to its ability to drive the transcription of the immunoglobulin kappa light chain genes in B cells.[30] Since that time, it has been found to drive the activation of several immunologically important genes including cytokines, acute phase response proteins, and cell adhesion molecules.[31,32,33] Among the myriad of NF-κB responsive genes are the APRs serum amyloid protein, angiotensinogen, C3 complement and complement factor B as well as the cytokines IL-1, IL-2, IL-6, IL-8, G-CSF, GM-CSF, TNF-α, TNF-β and interferon-β (IFN-β).[33] Additionally, the costimulatory molecule B7.1 is inducible through NF-κB.[34] Although NF-κB transcription has been shown to be induced by many proinflammatory and immunomodulatory molecules including IL-1, LPS, CD30 and CD40 ligands, TNF, lymphotoxin-α and lymphotoxin-β [35,36,37,38,39,40] we will be concentrating on the pathway as it pertains to the activation of the Toll receptor and its putative ligand. It should, however, be noted that the majority of the pathway is conserved or homologous between activating receptors.

The activation of NF-κB is regulated by its cytoplasmic inhibitor, IκB. Under normal circumstances IκB binds NF-κB in a manner that prevents the presentation of its nuclear localization signal thus sequestering NF-κB in the cytoplasm.[41,42] When the NF-κB pathway becomes activated, IκB is phosphorylated, ubiquinated and degraded by the

proteasome leading to dissociation from NF-κB.[43,44,45,46] In this manner the inhibitor is degraded and the newly released NF-κB can now translocate to the nucleus and activate transcription of its responsive genes.

Recently, a kinase complex, termed the signalsome, consisting of the NF-κB-inducing kinase (NIK) and two IκB kinases (IKKα and IKKβ) has been implicated in the phosphorylation of IκB.[47,48,49,50,51,52] NIK is thought to be the protein responsible for binding upstream regulatory elements while the IKKs have been shown to phosphorylate IκB on specific serine residues. Thus there exists a directly upstream mechanism by which IκB is phosphorylated, leading to its degradation and the activation of NF-κB.

NIK has been shown to interact with a family of proteins referred to as TNF-receptor associated factors (TRAF)[48,53] placing this family of proteins directly upstream in the pathway. The TRAFs are signal transducing proteins characterized by a conserved carboxy-terminal TRAF-C domain, an α-helical TRAF-N domain and an amino-terminal RING finger.[54,55] Recent evidence has implicated a member of the TRAF family involved in IL-1 and Toll-mediated NF-κB activation.[56] This novel protein is referred to as TRAF6.

TRAF6, in turn, has been found to interact with a serine/threonine kinase referred to as IL-1 receptor-associated kinase (IRAK).[55] However, the study that reported the cloning of IRAK did not show direct NF-κB activation by this newly discovered protein.[57] Nevertheless, a striking homology to the ser/thr kinase Pelle in *Drosophila* and experiments showing a coprecipitation with TRAF6 in IL-1 treated cells[55] as well as experiments showing that an antibody to IL-1RI will coprecipitate with IRAK and not

TRAF6 in IL-1 treated cells[55] implies that IRAK is instrumental in recruiting TRAF6 to the IL-R complex.

The final link in the pathway is the adapter molecule MyD88 which has been shown to interact with IRAK via homologous death domains.[58,59] Additionally, MyD88 contains a C-terminal TIR domain which has been shown to interact with the cytoplasmic domains of both the IL-1 receptor and Toll. Experimental evidence has shown NF-κB activation with overexpression of MyD88 as well as inhibition of Toll-activated NF-κB by a dominant negative version of the protein.[58] Thus, MyD88 provides the most upstream link in the signal transduction pathway of the IL-1R/Toll family which ultimately results in the activation of NF-κB responsive genes.

1.5 A Conserved Pathway

Perhaps the most striking element of this pathway is how remarkably it has been conserved over evolutionary time. Similar pathways exist not only in invertebrates, but recent evidence points to a link to the host defense systems of plants as well, placing conserved elements of this pathway in organisms separated by one billion years of evolutionary history. This conservation is a testament to the elegance and efficiency of this pathway.

1.5.1 *Drosophila*

The response of *Drosophila* and other insects to infectious challenge is the rapid synthesis of antimicrobial peptides in the fat body and hemocytes of the organism.[60] The genes that encode these proteins contain regulatory regions with multiple binding sites for

transcription factors of the rel family.[61,62] In fact, three members of this family, Dorsal, Dif and Relish, have been shown to become activated in response to infection.[63,64,65] As members of the rel family, these transcription factors all share homology with NF-κB.

Perhaps the signal transduction pathway of rel-related proteins in invertebrates is best understood in the case of the *Drosophila* Toll-Dorsal pathway. As stated before, Toll is a membrane-bound receptor that was first identified via its role in determining dorsal-ventral patterning in the *Drosophila* embryo.[23,24] Later studies implicated it in the induction of the antifungal peptide drosomycin.[27] Elegant double-mutant analysis has elucidated the order of action of many of the proteins involved in the Toll-Dorsal signaling pathway.[24]

The ligand of the Drosophila Toll-Dorsal pathway is a molecule called Spatzle, which itself is the result of proteolytic cleavage. Upon binding and activation of Toll, two intracellular proteins become recruited and activated. Tube is thought to function as an adapter protein and, thus, appears to be a functional analog of MyD88 in the NF-κB pathway. Tube is responsible for the recruitment of the serine/threonine kinase Pelle, which shares remarkable homology with IRAK, to the receptor complex. Pelle, in turn, complexes with a recently cloned intermediary dTRAF which, as its names suggests, is the likely *Drosophila* homolog of TRAF6.[66] Through mechanisms which have not been completely described, the activation of dTRAF results in the phosphorylation of IκB homolog Cactus. This phosphorylation results in the rapid degradation of Cactus. Released from the inhibition of Cactus, Dorsal translocates to the nucleus where it effects transcription of its responsive genes. Thus, Dorsal is the *Drosophila* homolog of the mammalian NF-κB.

1.5.2 Plants

Several reviews on plant host defense mechanisms have recently been published which show a remarkable conservation between components of the mammalian NF-κB and Drosophila Toll-Dorsal pathways and the products of plant resistance (R) genes.[67,68] Conserved domains found include the TIR domain, the LRR domain and serine/threonine kinases homologous to IRAK and Pelle. Particularly striking is the tobacco N protein which contains not only a LRR domain homologous with the extracellular portion of the Toll receptor but also the TIR domain, which is present on the cytoplasmic portion of Toll.

Of particular interest is a group of R genes bearing resemblance to the serine/threonine kinases IRAK and Pelle. Members of this group include the tomato proteins *pto, pti*, and *fen*. This has led to the classification of these homologous proteins in a group referred to as serine/threonine innate immunity kinases (SIIK).[63] The remarkable homology that exists between these molecules and other components of this pathway is summarized in Figure 1.

The conservation of major portions of host defense mechanisms across organisms separated by more than 1 billion years of evolution has great significance. Of perhaps greater interest is the conclusion that mammals have co-opted this ancient system of innate immunity as a trigger for antigen-specific adaptive immunity through the induction of costimulatory molecules.[69]

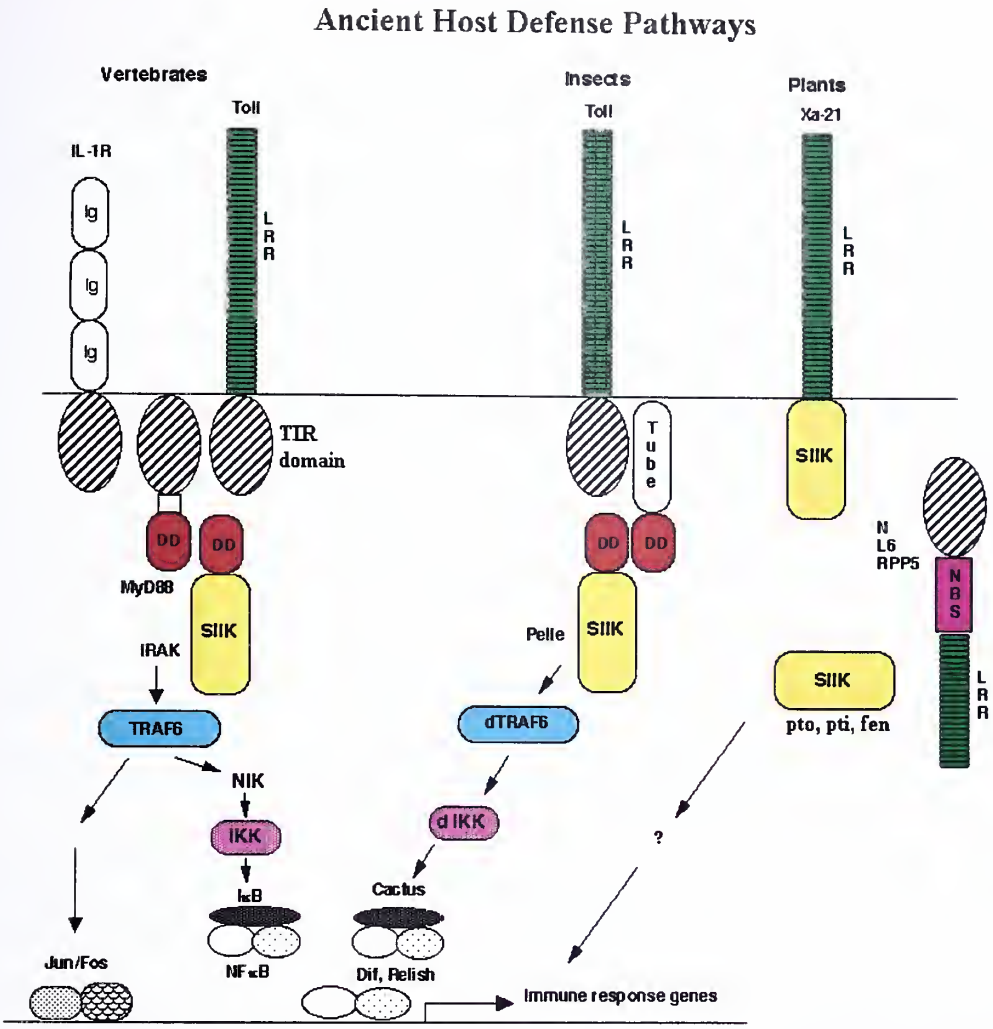

Fig. 1 A schematic representing conserved elements of host defense. On the left, the vertebrate system signals through two distinct receptors, the IL-1 receptor and the Toll receptor, both of which posses a homologous Toll/IL-1R (TIR) domain. The adapter molecule MyD88 complexes with the receptor via its own TIR domain as well as with the serine/threonine innate immunity kinase (SIIK), Il-1 receptor-associated kinase (IRAK) through homologous death domains (DD). IRAK then signals through TNF receptor-associated factor 6 (TRAF6) which, in turn activates a kinase complex consisting of NF-κB inducing kinase (NIK) and two IκB kinases (IKK). Thus IκB becomes phosphorylated, its degradation is signaled and the transcription factor NF-κB translocates to the nucleus where it activates genes important in host defense. The insect pathway, represented by the Drosophila Toll receptor is depicted in the middle. The adapter protein Tube performs a similar function as the mammalian MyD88. Similarly, Pelle is a SIIK with a function similar to that of IRAK. DTRAF is the Drosophila homologue of TRAF6 and was recently described. Cactus is the cytoplasmic inhibitor of the rel related proteins Dorsal, Dif, and Relish which can induce the transcription of host defense genes, specifically those that encode for antimicrobial peptides. The mechanisms of host defense in plants are less clear. Nevertheless, there exist proteins, products of plant resistance (R) genes that share extensive homology with components of vertebrate and invertebrate host defense. For example the tomato pto, pti, and fen genes encode SIIKs homologous to IRAK and Pelle. The tobacco N protein, the flax L6 protein and the arabidopsis RPP5 all contain leucine rich regions (LRR) and domains similar to the intracellular portion of Toll (TIR). However, contrasting the membrane-bound receptors of vertebrates and invertebrates, these proteins are expressed intracellularly. The Xa-21 rice protein is a membrane-bound molecule with an extracellular LRR and a cytoplasmic SIIK domain.

1.6 IRAK

IRAK is a 712-amino acid SIIK that was first reported in 1996.[57] The protein was first obtained by coimmunoprecipitation with the IL-RI from embryonic kidney epithelial cells (293 cells) that were stimulated with IL-1. Micropeptide sequencing, construction of PCR primers, amplification of a cDNA probe and screening of a teratocarcinoma cDNA library resulted in the cloning of the full-length gene. Sequence analysis revealed a putative kinase domain (residues 208-524) with 12 subdomains and 15 highly conserved amino acids suggestive of a protein kinase.[70] This kinase domain shares extensive homology with the tomato kinase pto (30%)[71] and *Drosophila* Pelle (32%). Additionally, IRAK and Pelle are 50% homologous in a region spanning kinase subdomains IV to VII. Note that this relatedness is significantly greater than that which exists between IRAK and its closest mammalian protein, human mixed-lineage kinase (25%).[72] Also of interest is the amino-terminal death domain which shares weaker homology with the corresponding region in Pelle. It is this death domain that is required for the interaction between IRAK and MyD88.[58] Fig. 2.

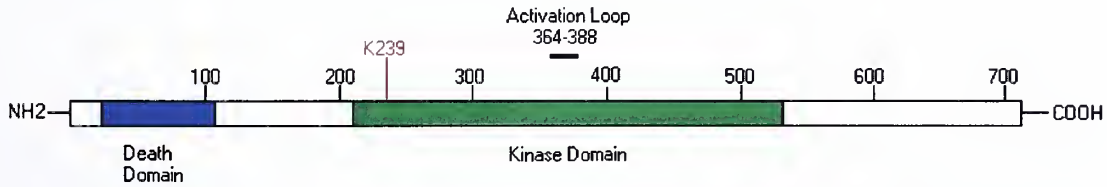

Fig. 2 Schematic of the IL-1 receptor-associated kinase (IRAK). IRAK is a member of the family of serine/threonine innate immunity kinases (SIIK). Its key features are an amino-terminal death domain which shares homology with a similar region on the adapter molecule MyD88 as well as a kinase domain spanning residues 208-524. Of the several highly conserved kinase residues, our target for creation of a kinase deficient protein was the lysine at position 239, marked in red.

The study that originally described IRAK also provides some clue as to its function. It was shown that IRAK/IL-1R interaction is IL-1 dependent and that IRAK

becomes highly phosphorylated upon activation.[57] A more recent study has shown, by virtue of nonspecific kinase inhibition, that IRAK/IL-1RI complex formation is not dependent upon phosphorylation and that the phosphorylation of IRAK is likely a signal for its degradation by the proteasome, in a similar fashion to IκB.[73] Nevertheless, there has been no successful attempt to induce a more than 3 fold increase in NF-κB activity through overexpression of IRAK. As such, it is difficult to describe the role of IRAK in NF-κB signal transduction. We postulate that in order for IRAK to activate NF-κB, it must first be recruited to the cell membrane. Here we report the construction of a NF-κB-activating form of IRAK and evidence that may help explain how it functions in vivo.

2. Statement of Purpose

The aim of this study is to establish the molecule IRAK as a participant in signal transduction of the Toll/IL-1R/NF-κB pathway. Additionally, we seek to describe the organizational structure and interaction of other proteins involved in this cascade.

3. Materials and Methods

3.1 Constructs

Reasoning that translocation to the cell membrane is required for activation of IRAK, a chimeric molecule containing the extracellular domain of CD4 fused to IRAK was created. Full length IRAK was obtained from E. Kopp. Full length CD4 was obtained from R. Medzhitov. The extracellular domain (residues 1-387) of CD4 was generated by PCR incorporating a downstream EcoRI site. This fragment was then subcloned into the vector pCINEO. IRAK (residues 8-712) was generated by PCR

incorporating an upstream EcoRI site. This fragment was then subcloned into the vector pCINEO. The IRAK fragment was then subcloned in-frame downstream of the CD4 fragment in pCINEO utilizing the engineered EcoRI site. A kinase deficient mutant of IRAK was made using PCR based site-directed mutagenesis to change the lysine residue at position 239 to alanine resulting in the IRAK K239A mutant. (Fig. 2) A chimeric molecule incorporating the extracellular portion of CD4 was then constructed in a similar fashion as described previously.

A death domain deleted version of this molecule was also created by using downstream PCR primers resulting in a CD4/IRAKC molecule which includes the IRAK residues 107-712. A similar death-domain deleted version of CD4/IRAK239 was created analogously and called CD4/IRAK239Δ. Included are IRAK residues 152-712. The constructs CD4/Toll (intracellular signaling domain of Toll fused to extracellular CD4), MyD88, TRAF6 and a dominant negative version of TRAF6, TRAF6C, all separately subcloned in pCINEO, were kindly provided by R. Medzhitov.

3.2 Stable Cell Line

A cell line consisting of the human embryonic kidney epithelial cells (293 cells) was stably transfected with a luciferase reporter system inducible by an NF-κB activated promoter. This cell line is referred to as 293Luc and was utilized to determine relative NF-κB activation of various components. Kindly provided by E. Kopp.

3.3 Assay

For each assay, appropriate quantities of DNA and the transfection reagent FuGene 6 reagent (Boehrengier Manheim, Inc.) were used to transfect ~2 x 10^5 293Luc cells in 2ml DMEM/10%FCS media in 35mm wells. The cells were allowed to grow overnight and were harvested, washed in 1ml PBS buffer and lysed with lysis buffer (Luciferase Assay Kit, Promega Corp.). An aliquot of 20 µl of lysate was then tested for luciferase activity with the addition of 100 µl luciferase substrate (Luciferase Assay Kit, Promega Corp.) and measured in a luminometer (Lumat LB 9501, Berthold Corp.). Results were standardized to a control transfection of vector (pCINEO) alone.

3.4 Antibody

All antibody used was IgG and was kindly provided by G. Losyev.

4. Results

4.1 NF-κB Activation

Although IRAK has previously been shown to coimmunoprecipitate with IL-1RI in 293 cells treated with IL-1 in a phosphorylation-dependent manner[57] it has never been shown to significantly induce NF-κB activation. To assess the ability of IRAK to drive NF-κB promoters, we transiently transfected 293 Luc cells with the chimeric molecule CD4/IRAK. As shown in Fig. 3, CD4/IRAK induced NF-κB activation in a dose-dependent fashion.

To test whether or not kinase activity was necessary for IRAK to induce NF-κB, we transiently transfected 293 Luc cells with the CD4/IRAK239 kinase deficient construct. As with wild type, CD4/IRAK239 induced NF-κB in a dose-dependent fashion.

Activation was significantly less than that of wild type. Additionally, there was a plateau effect of NF-κB activation by CD4/IRAK239 at higher concentrations.

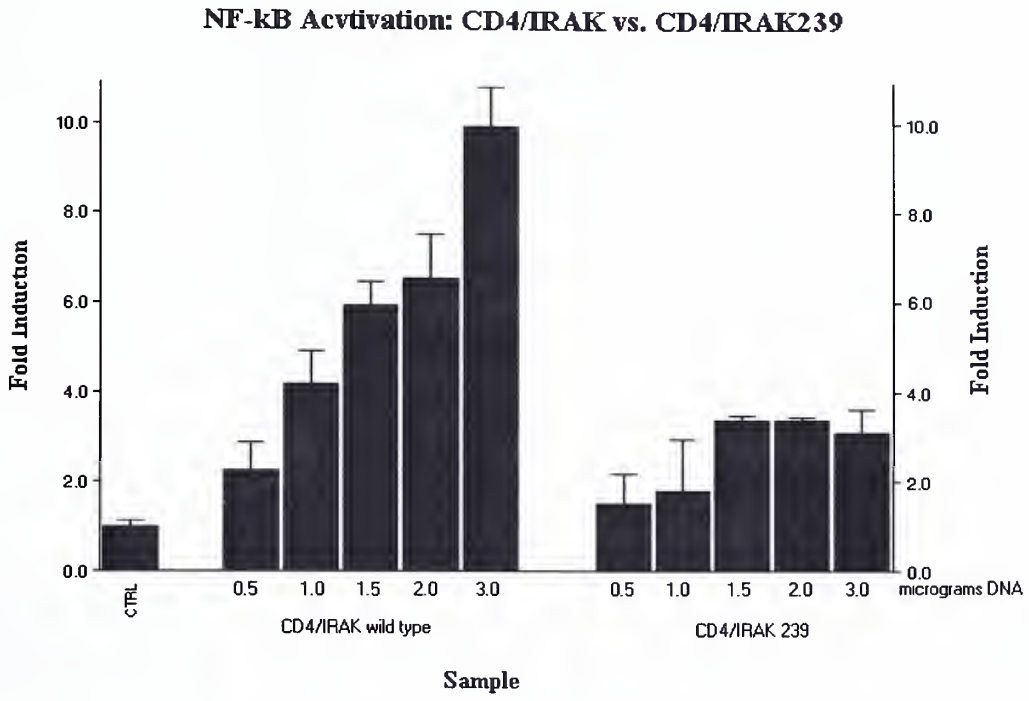

Fig. 3 Relative NF-κB Activation of CD4/IRAK constructs. Both CD4/IRAK contructs were transfected into 293 Luc cells. Both wild type and K239A did activated NF-κB in a dose-dependent fashion. NF-κB activation by CD4/IRAK239 was significantly less than that of wild type. Also, there was a blunting effect on NF-κB activation by CD4/IRAK239 at higher concentrations of transfected DNA.

We also wanted to assess the ability of a dominant negative version of TRAF6, TRAF6C, to inhibit NF-κB activation by both wild-type and 239. Thus, either wild type or 239 DNA was cotransfected with TRAF6C into 293 Luc cells. The NF-κB activation of both was inhibited by TRAF6C. Fig. 4.

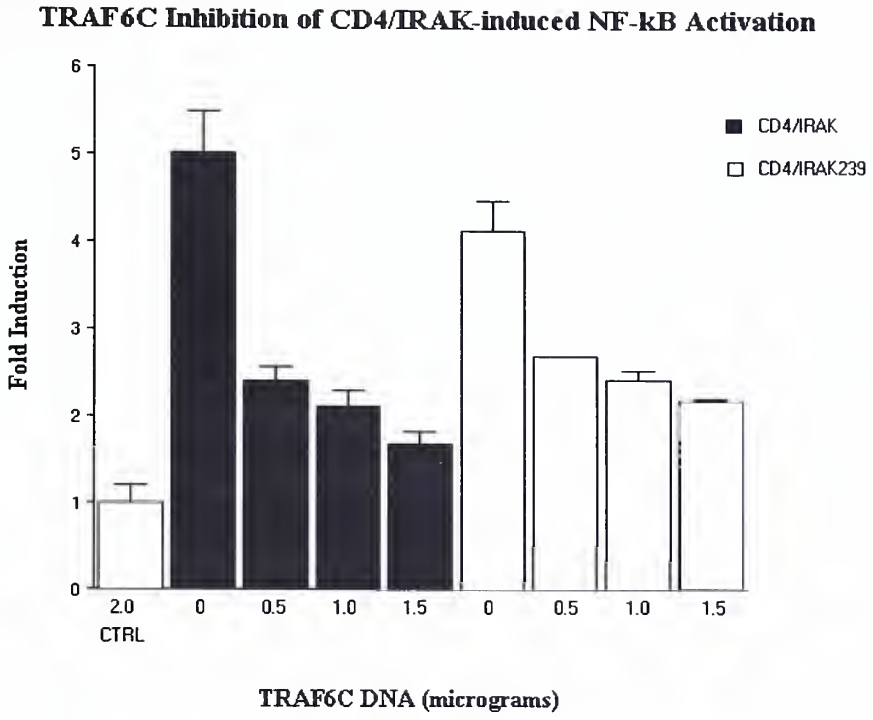

Fig 4. Inhibition of NF-κB activation by TRAF6C. 293 Luc cells were transfected with 1.5 μg of either CD4/IRAK wild type or CD4/IRAK239. These cells were also transfected with TRAF6C, a dominant negative version of TRAF6, in various concentrations. Both CD4/IRAK and CD4/IRAK239 activated NF-κB as expected. Both were inhibited by TRAF6C in a relatively equivalent manner.

Furthermore, we were interested in the nature of the interactions between proteins in the NF-κB pathway. In order to assess this, chimeric molecules containing the extracellular portion of CD4 and the IRAK protein with the death domain deleted were created. Both wild type death domain deleted (CD4/IRAKC) and K239A death domain deleted (CD4/IRAK239D) were constructed. Reasoning that the death domain is important for determining proper protein alignment via the MyD88 molecule we theorized that deletion of the death domain would result in lower activation. Additionally, assuming IRAK underwent autophosphorylation which would result from the close proximity of at least two IRAK proteins we attempted to induce NF-κB

activation by the death domain deleted molecules by cross-linking their extracellular CD4 receptors with αCD4 IgG. Fig. 5.

Both death domain deleted molecules activated NF-κB to a lesser extent than the full length proteins. Additionally, while cross-linking full-length CD4/IRAK with αCD4 IgG provided minimal additional activity over control cross-linking with αCD8 IgG, both CD4/IRAKC and CD4/IRAK239D activated NF-κB at a significantly higher level with αCD4.

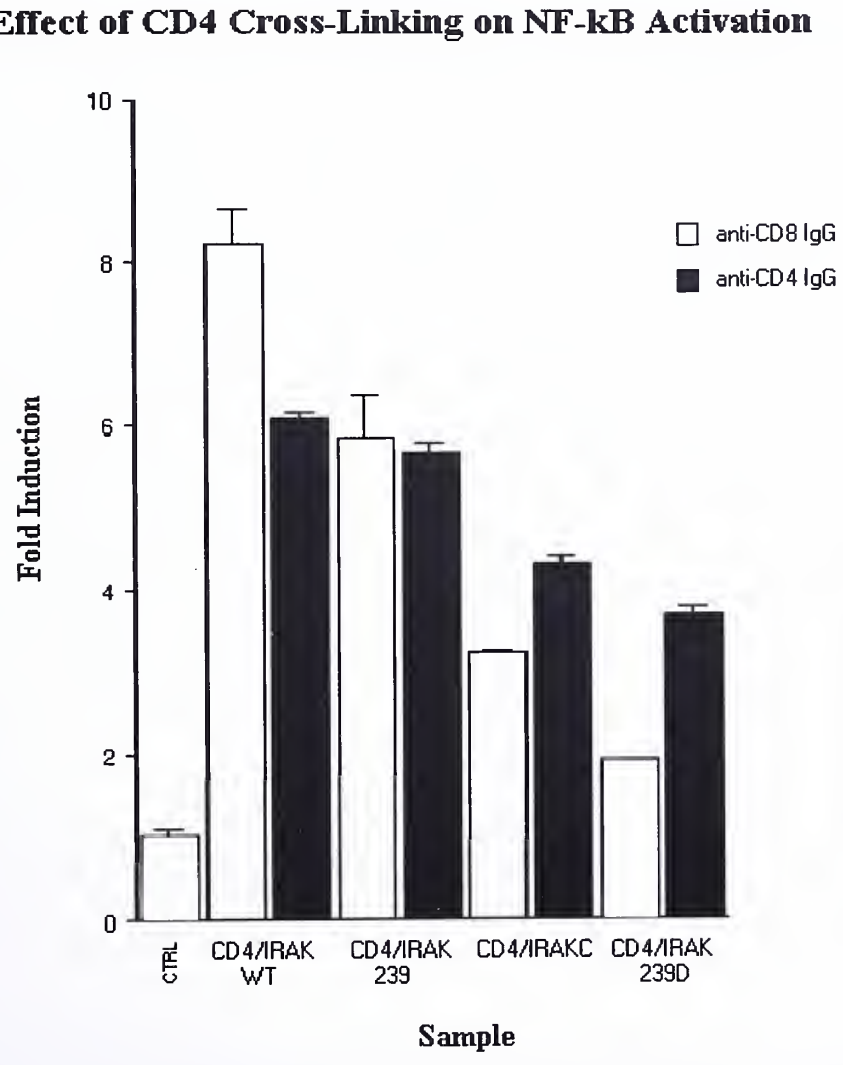

Fig. 5 Effect of cross-linking with αCD4 Ab on CD4/IRAK-induced NF-κB activation. 293 Luc cells were transfected with 2.0 μg of DNA. 18 hours later, all samples were cross-linked by either αCD4 or αCD8 Ab. 5 hours subsequently, cells were harvested and lysed. Lysates were measured for luciferase activity. There was no potentiation of NF-κB activation by αCD4 on full length CD4/IRAK molecules, either wild type or 239. There was an approximately 2-fold potentiation of NF-κB activation by αCD4 on death domain deleted CD4/IRAK molecules, both wild type and 239. Overall, there was less actvity in the death domain deleted molecules vs. the full length molecules. Cross-linking increased activation but it was still slightly less than that of full-length for both wild type and 239.

Also of interest was the method by which Toll became activated which might reveal useful information regarding the nature of the Toll ligand. To address this question, we transfected 293 Luc cells with CD4/Toll and attempted to dimerize the receptors using αCD4 IgG. Fig. 6. The result was striking. CD4/Toll, as expected, induced NF-κB activation in the control αCD8 cross-linking. However, when we added a dimerizing ligand (αCD4) for this chimeric molecule, induction of NF-κB was significantly potentiated.

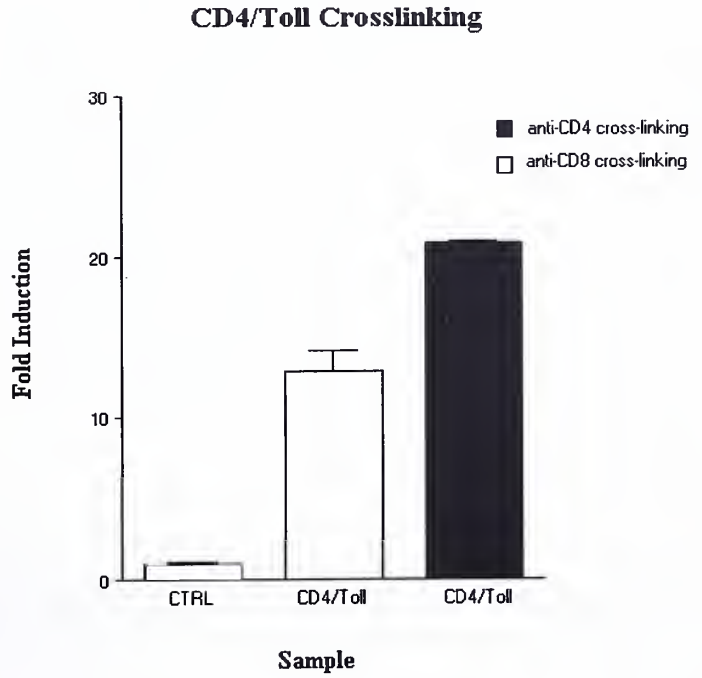

Fig. 6. NF-κB activation by CD4/Toll is induced by cross-linking the receptor with αCD4 IgG. 293 Luc cells were transfected with 2.0 μg of CD4/Toll. 18 hours after transfection they were treated with

either αCD4 IgG or αCD8 IgG. Cells were then harvested 5 hours later and assayed for luciferase activity. The sample treated with αCD4 showed significantly more activity.

4.2 AP.1 activation

In addition to the NF-κB pathway, Toll is known to signal through another transcription factor, AP.1. To assess whether IRAK is part of this signaling pathway we cotransfected 293 cells with an AP.1 luciferase reporter gene and several proteins involved in Toll/NF-κB activation. Fig. 7. Both CD4/Toll and CD4/IRAK mildly activated AP.1 while TRAF6 had a much more significant effect.

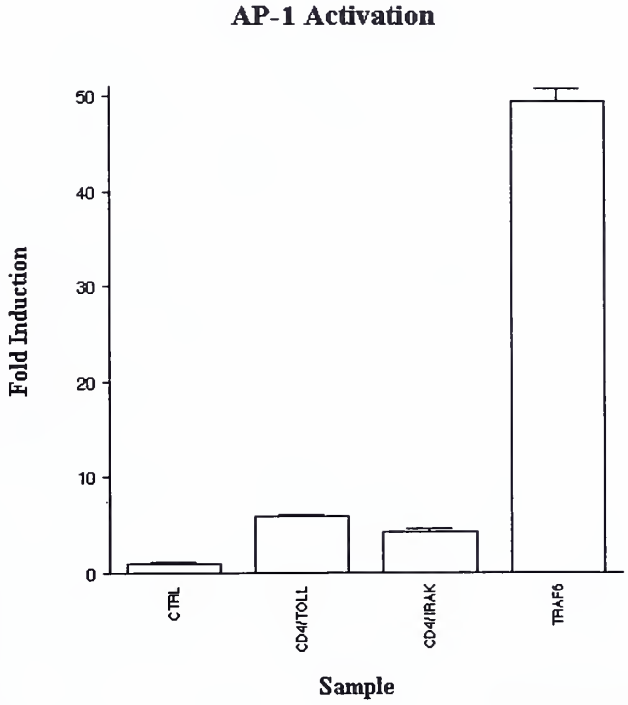

Fig. 7. Ap-1 Activation. 293 Luc cells were transfected with 2 μg of DNA. They were then harvested 24 hours later and assayed for luciferase activity. Both CD4/Toll and CD4/IRAK moderately activated the AP-1 pathway while TRAF6-induced activation was significantly greater.

5. Discussion

5.1 IRAK

Although there have been experiments that showed coimmunoprecipitation of IRAK with components of the IL-1R complex upon stimulation with IL-1 and that IRAK shares extensively homology with kinases involved in host defense of other organisms[57], there has not been a successful attempt to show significant NF-κB induction with IRAK. Here we show an almost 10-fold activation of NF-κB by our CD4/IRAK construct. Our data indicate that IRAK is indeed a SIIK involved in NF-κB signal transduction. Additionally, inhibition of IRAK-induced NF-κB activation by a dominant negative version of TRAF6 places IRAK upstream of TRAF6 in the pathway. Sequence analysis of IRAK reveals a death domain region which is homologous to a similar region on the adapter molecule MyD88 suggesting that MyD88 is the immediate upstream protein in the cascade. Experiments were also done showing that a dominant negative version of IRAK is capable of inhibiting MyD88-induced NF-κB activation.[58] Thus, we conclude that IRAK is a serine/threonine innate immunity kinase involved in signal transduction of the NF-κB pathway. Additionally, IRAK is involved in the pathway between the proteins MyD88 and TRAF6.

As shown, we were able to induce NF-κB activation with both CD4/IRAK wild type and CD4/IRAK239. This suggests that kinase activity is not absolutely necessary for IRAK function. Interestingly, this is similar to another known SIIK, receptor-interacting protein (RIP) which functions in the TNF receptor pathway. RIP possesses putative kinase domains but has been shown to induce NF-κB activity independent of the proper function of those domains.[74]

It is known that IRAK complexes with MyD88 upon activation of the upstream receptor prior to its phosphorylation.[73] That same study also showed that phosphorylation

is likely a target for the degradation of IRAK via the proteasome. It seems likely then, that the kinase activity inherent in IRAK may represent a regulatory mechanism to prevent the overexpression of immunomodulatory peptides by the NF-κB pathway. Sepsis results from a massive increase in vascular permeability whose sequelae include high-output cardiac failure and end-organ perfusion insufficiencies. The initial insult, increased vascular permeability, is an evolutionarily adaptive response mediated by cytokines, predominantly TNF-α and IL-1.[16] This increased permeability is necessary to allow the effector cells and molecules of host defense access to the nidus of infection. When that system is overexpressed, sepsis results. The pathways of TNF-α and IL-1 potentiation are mediated by similar SIIKs: RIP and IRAK, respectively. The data suggest that kinase activity is unnecessary for signal transduction for both of them yet that activity remains conserved over a billion years of evolution. Therefore, an adaptive function for this activity must exist. One model suggested by the data is that IRAK is recruited to the receptor complex independent of phosphorylation, transduces its signal to TRAF6 in an as yet undetermined manner, rapidly autophosphorylates and targets itself for destruction preventing overexpression of TNF-α and IL-1. If the signal is indeed independent of phosphorylation, as the data here suggests, simply dephosphorylating IRAK cannot turn off the signal. Therefore, degradation becomes the autoregulatory mechanism.

Interestingly, the level of NF-κB activation induced by kinase deficient IRAK (CD4/IRAK239) lags behind that of wild type (CD4/IRAK). Additionally, the increase in NF-κB activation with an increase in amount of DNA transfected becomes blunted at higher levels of CD4/IRAK239. If we expect that kinase activity is required only for its

role in degradation and autoregulation we would suspect that kinase knockout molecules might potentiate NF-κB activation. Yet, this is not borne out by the data. Almost counterintuitively, NF-κB activation decreases at higher concentrations of CD4/IRAK239. This suggests an as yet undiscovered function of IRAK kinase activity. This finding in the data is not inconsistent with the conclusion that IRAK functions as a homodimer that autophosphorylates. Perhaps only one of the two molecules in the dimer requires phosphorylation. At lower concentrations of CD4/IRAK239, endogenous wild type IRAK is recruited to the CD4/IRAK239 molecule forming a heterodimer wild type/K239A complex, phosphorylates itself or its partner protein and activates NF-κB. As the concentrations of K239A increase, fewer heterodimers are formed and more homodimer CD4/IRAK239 molecules spontaneously aggregate. Because neither one of the proteins in this complex possess kinase activity, the signal is disrupted and activation declines.

There is additional evidence to suggest that IRAK does indeed function as a dimer. The experiment with the death domain deleted molecules provides some valuable insight. Because IRAK complexes with MyD88 via the death domain, deletion of the death domain would most likely remove the mechanism by which IRAK would aggregate should such a mechanism exist. That the death domain deleted mutants, both wild type and 239, were less effective in NF-κB induction is consistent with the notion that there is some protein superstructure that potentiates signal transduction and that superstructure is mediated through interactions involving the death domain. When we cross-linked the death domain deleted molecules, there was a significant increase in activation whereas the full length molecules saw little if any potentiation of signal. Because αCD4 IgG

would be expected to dimerize these molecules, it seems likely that the natural activated state of IRAK is a homodimer and that conclusion is suggested by our results from this experiment.

Thus we are left with two seemingly incongruent conclusions. On the one hand we have activation by kinase deficient molecules, which is consistent with at least one other SIIK. On the other hand we have a lower level of activation versus wild type and declining activation with increased concentration of CD4/IRAK239. This suggests the possibility that kinase activity may be important for signal transduction purposes. As we have stated before, the closest mammalian homologue of IRAK is human mixed lineage kinase which itself is more closely related to tyrosine kinases than serine/threonine kinases. Additionally, IRAK shares more homology with SIIKs in invertebrates and plants than it does with other mammalian kinases.[57] This suggests that the SIIKs are a semi-distinct class of kinases that evolved very early on. As such, one might expect unique structural and functional features associated with this class of proteins. The degradation of IRAK might be one such feature. Most kinases can rapidly undergo dephosphorylation by phosphatase molecules that results in inactivation. There has been no evidence to suggest such a mechanism exists for IRAK. Indeed, it seems that the only mechanism for the inactivation of IRAK is its phosphorylation-dependent degradation.

One possible explanation for our seemingly contradictory evidence is that kinase activity in IRAK and perhaps in all SIIKs has a dual role. It has a well established function in degradation with autoregulatory implications and may well have a role in signal transduction also. Although there is no known substrate for IRAK kinase activity other than itself,[75] it is not inconsistent with the data to suggest that IRAK either

autophosphorylates or cross-phosphorylates when dimerized which might induce conformational changes and allow the binding of TRAF6 or other downstream molecules. This is consistent with our data that shows mild activation by CD4/IRAK239 that decreases at high concentrations. While we would expect that activation should be markedly decreased by the failure of IRAK239 to phosphorylate itself to induce this assumed binding event there is a counterbalance effect by the mutation's ability to inhibit degradation and the ability of CD4/IRAK239 to recruit wild type IRAK into a protein dimer. Presumably, one molecule of wild type IRAK in a dimer would retain some ability to activate. As we raise the concentration of kinase deficient IRAK we increase the probability that the IRAK dimers will now consist of two molecules of CD4/IRAK239 which, without any kinase activity, would terminate signal transduction and result in a decrease in NF-κB activation.

The question of why this system of degradation-for-deactivation evolved still remains unanswered. Presumably, IRAK, when phosphorylated, can bind downstream molecules including TRAF6 and others which remain unknown at present. Perhaps the formation of this complex is very energetic and simply dephosphorylating IRAK does not provide enough instability to cause spontaneous disruption. Perhaps the answer is simply that the SIIKs evolved prior to the evolution of phosphatases and never faced selective pressure to adapt. Nevertheless, the search for downstream molecules and their interaction with IRAK remains an important avenue of study.

5.2 Toll

Although there is recent data that implicates LPS in the activation of the mammalian Toll receptor[29], the search for the ligand remains active. We know that the ligand for *Drosophila* Toll is spatzle, which itself is generated by an endogenous proteolytic reaction.[63] Thus, the possibility exists that the ligand for mammalian Toll is an endogenous signal.

In order to gain some insight into the function of Toll we transiently transfected 293 Luc cells with a chimeric molecule consisting of the extracellular portion of CD4 and the signaling domain of Toll. After cross-linking with αCD4 IgG, NF-κB activation was potentiated by a factor of two. This suggests that Toll functions in a dimer, binding one molecule of MyD88. In this model, the TIR domain trimerizes, two domains from the intracelluar portion of Toll and one from the MyD88 protein. The implication is that the ligand for Toll may have two identical epitopes capable of binding the same region on two Toll molecules. Fig. 8

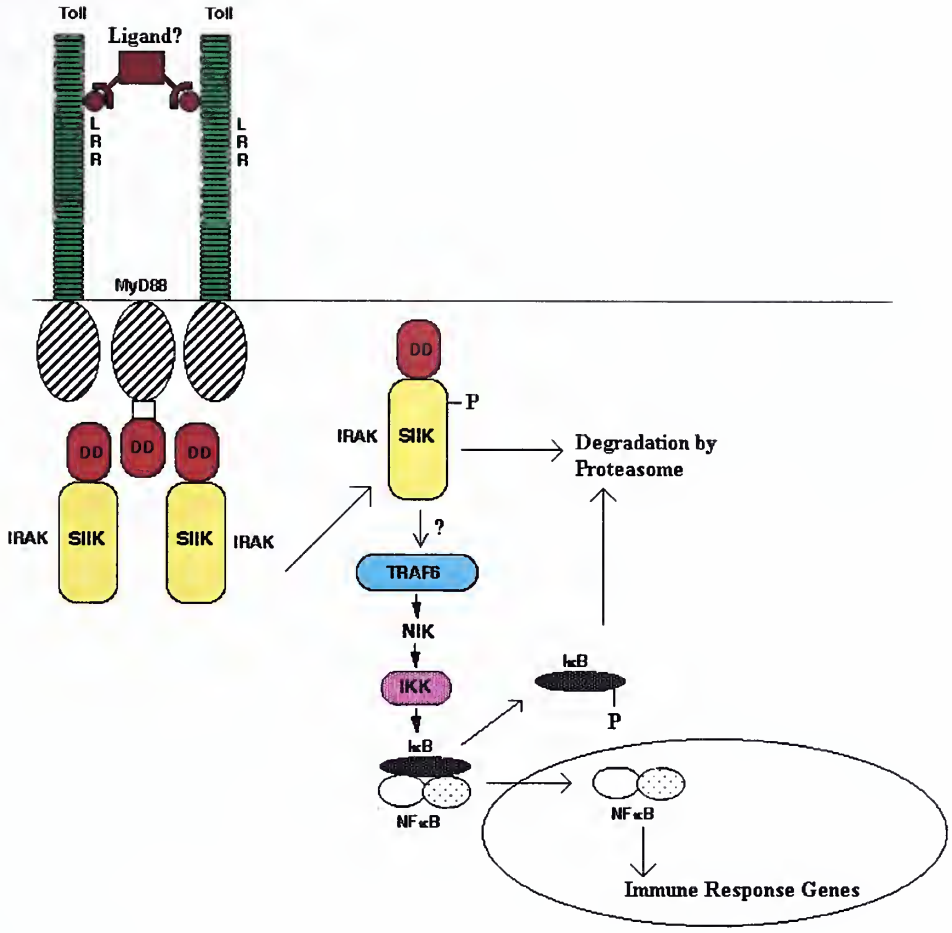

Fig. A schematic representation of the organization of the Toll/NF-κB pathway. The data is consistent with a potentiation of NF-κB activation when Toll is dimerized. In such a complex, one MyD88 molecule would then associate with the dimerized Toll complex forming a TIR domain trimer. Then a death domain trimer would link the one MyD88 molecule with two molecules of IRAK. Again, the data from the death domain deleted experiments is consistent with potentiation of NF-κB activation by IRAK dimerization. The IRAK dimer then autophosphorylates or cross-phosphorylates which is likely to have a dual role: 1) it signals for the degradation of IRAK by the proteasome and 2) it induces a conformational change which allows IRAK to transduce the activation signal to downstream molecules. The end result is the phosphorylation of IκB and the translocation of NF-κB to the nucleus where it induces the transcription of immune response genes.

Implicit in the assumption that the ligand possesses two identical epitopes is the high probability that such a ligand would be endogenous. The model for such a receptor and ligand is the receptor for platelet-derived growth factor (PDGF).[76] The PDGF receptor is a receptor tyrosine kinase capable of autophosphorylation when it becomes dimerized. Of important consequence is that the ligand for this receptor possesses at least two

binding epitopes with a regular structure. This is true for any receptor that forms dimers. This ligand is likely itself to be the product of an endogenous process. LPS does not possess such symmetry and our data would be inconsistent with an assertion that LPS is the ligand for Toll. Nevertheless, there is evidence that suggests LPS is involved in Toll activation.[29] It is likely that there exists an intermediate step between initial recognition of LPS and activation of Toll and that this step generates the ligand for the Toll receptor.

Although it may appear that activation in the absence of cross-linking the Toll receptor is higher than that which would be predicted by our model, it is likely this is the result of experimental design. By transfecting large quantities of CD4/Toll, the TIR domain becomes overexpressed. In such a state, the rate of spontaneous dimerization of the CD4/Toll molecule increases. Thus, as is consistent with the data, activation will increase despite the fact that we have designed no specific mechanism for receptor dimerization.

5.3 AP-1

Interestingly, the AP-1 pathway has been shown to become activated by the Toll receptor but remain quiescent with IL-1R activation.[77] Because these two receptors share an NF-κB activation pathway, it was interesting to discover that components of this pathway can mildly (Toll, IRAK) and significantly (TRAF6) activate AP-1. This suggests an alternative signaling mechanism by which the AP-1 pathway distinguishes between Toll and IL-1R activation. The data is consistent with the notion that this discrimination does not occur at the level of IRAK or TRAF6 as both can induce activation of AP-1.

6. Conclusions

The link between innate and adaptive immunity has been avidly pursued for several years. Because of its central role in the induction of host defense responses, the NF-κB pathway has occupied much of that attention. Many questions regarding the function of the NF-κB pathway and its constituent proteins have been answered. Nevertheless, many questions remain.

One area that bears further study is the function of IRAK. Specifically, what is the role of kinase activity in signal transduction? We have shown here that kinase activity is not strictly necessary for NF-κB activation yet we also have data that suggests it may play a part. Does IRAK complex with downstream molecules in a phosphorylation-dependent process? Is there an intermediate molecule between IRAK and TRAF6 involved in this putative protein complex? Is the function of IRAK unique or is it shared by other SIIKs, implying that this is a novel class of proteins with properties not shared by other kinases?

Of significant importance is the elucidation of the mechanism of induction of costimulation. Is the NF-κB pathway uniquely responsible for inducing costimulation? Studies examining the role of each of the constituent molecules of NF-κB in induction of costimulation would be very useful as would experiments designed to elucidate other pathways/receptors with the ability to initiate an adaptive immune response.

The implications for these types of studies are far-reaching. As we develop our understanding of how innate and adaptive immunity are linked, either through NF-κB or

other mechanisms, our understanding of an enormous variety of pathological states increases. Additionally, the potential to treat these disease processes also grows.

One area that has attracted much attention is the manipulation of host defense elements for the treatment of cancer. Specifically, there have been efforts to develop a dendritic cell vaccination for melanoma. Malignant melanoma seems an ideal target for vaccination because its cells expresses an array of unique antigens, including the MAGE (for melanoma antigen) proteins first describe in association with melanoma in 1991.[78] As our understanding about the role and induction of B7.1 and B7.2 in the initiation of an adaptive immune response grows, perhaps we will be able to manipulate it to maximize a host defense response against these MAGE proteins or other self-peptides expressed only on malignant cells.

Conversely, the spectrum autoimmune disease poses a different problem. A single feature links these diseases. All are manifestations of an immune response that has become activated against self-antigens. Many of these diseases remain a mystery as to their etiology. Some have clear associations with a previous infection, i.e. poststreptococcal glomerulonephritis. Some, including multiple sclerosis, which is the result of a host defense response directed against the myelin-producing schwann cells of the nervous system, are less well understood. Perhaps future study into the function of NF-κB and the induction of costimulation can provide not only insight into the etiology of autoimmunity but also a method to develop host tolerance for self-peptides.

In addition to these examples and that of sepsis highlighted earlier, the understanding of host defense has remarkable implications in a wide variety of other fields. Perhaps one

day, the manipulation of our own immune systems will be a therapy as commonplace as the prescription of antibiotics.

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